DEVELOPMENT AND EVALUATION OF SOME NOVELFORMULATIONS FOREFFICIENT DELIVERY OF CHOLECALCIFEROL

A

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By

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1. **Proposed Title of the Research Work**

Development and Evaluation of Some Novel Formulations for Efficient Delivery of Cholecalciferol

2. Brief Introduction about the Research Area

Vitamin D has been recognized as an essential nutrient for bone health. Its deficiency is a global concern [1]. As per International Osteoporosis Foundation report, in North India, 96% of neonates, 91% of healthy school girls, 78% of healthy hospital staff and 84% of pregnant women were found to be suffering from Vitamin D deficiency while in south India 40% of males and 70% of females were observed with deficiency [2]. In a study from Europe carried among women aged over 80 years, the prevalence of 25(OH)D inadequacy was observed to be 80.9% [3]. The deficiency is prevalent in both urban and rural areas and among all socioeconomic and geographical strata [4]. Reduce exposure to sunlight is the main reason for its deficiency as ultraviolet-B (UV-B, 280-315 nm) irradiation of 7-dehydrocholesterol in skin epidermal cells is the primary source of vitamin D [5]. Vitamin D is an essential micronutrient owing to its ability to maintain calcium and phosphorus concentration at the normal range by enhancing the efficiency of the small intestine to absorb these minerals from the diet. The deficiency of vitamin D leads to rickets, osteomalacia, hyperparathyroidism and osteoporosis [6,7]. The cardiovascular mortality has also been reported in type 2 diabetes mellitus patients with vitamin D deficiency [8]. The regular dose of vitamin D (about 2000 IU/d) was reported to reduce the risk of developing type 1 diabetes as its low levels are associated with insulinemia and glucose intolerance [9]. The efficiency of vitamin D analogues as an anticancer agent has also been

reported due to its ability of inhibition of angiogenesis [10]. In such critical situations, intake of vitamin D has become essential.

Vitamin D is an oil-soluble micronutrient that is essential in the human diet for maintaining good health. It has two major chemical forms: vitamin D2 (ergocalciferol) and D3 (cholecalciferol) [11]. Vitamin D3 has more potency than vitamin D2 in humans and it is usually synthesized in the skin after exposure to light. Vitamin D3 may exist in a number of different chemical forms depending on environmental conditions, *e.g.* calciol, calcidiol, and calcitriol. Calcitriol (25-dihydroxyvitamin D3) is the biologically active form of vitamin D3 and controls calcium and phosphorus homeostasis, intestinal transport, bone metabolism, and renal calcium reabsorption, blood pressure, and insulin secretion [12].

Vitamin D3 deficiency often occurs in people who are not exposed to sufficient sunlight, and in individuals with metabolic (e.g. obesity or hyperparathyroidism) or gastrointestinal (e.g. celiac or inflammatory bowel disease) disorders [13]. Malfunction of the regulation of calcium and phosphorus absorption and bone metabolism can lead to osteoporosis and osteomalacia (pore formation and softening of bones) in adults, while rickets may occur in children in developing countries. Vitamin D sources are fairly limited in foods, and include products such as beef liver, dairy products, egg yolk, and fish [14]. Therefore, there is a high demand to enrich food and beverages with vitamin D3. Cholecalciferol is highly sensitive to environmental stresses (such as light, heat, and oxygen) and can easily be oxidized, leading to loss of its functionality and physiological benefits [15]. Therefore novel formulation strategies are required for the efficient delivery of cholecalciferol (Fig. 1).

3. Relevant state of the art Literature Review

It has been widely accepted that vitamin D deficiency (VDD) is a global health problem that impacts not only musculoskeletal health but also varied acute and chronic diseases [16]. Low vitamin D has been associated with an increased risk of diabetes mellitus, cardiovascular disease, certain cancers, cognitive decline, autoimmune disorders and pregnancy complications [17]. It has been estimated that 20 to 80% of US, Canadian and European men and women are vitamin D deficient. In Middle East and Asia, VDD is highly prevalent in both children and adults [18]. Even in India, numerous studies across various regions of the country indicate that approximately 70-90% of apparently healthy population is vitamin D deficient. Low vitamin D status is prevalent irrespective of age, sex, profession, rural/urban settings or regional distribution [19].

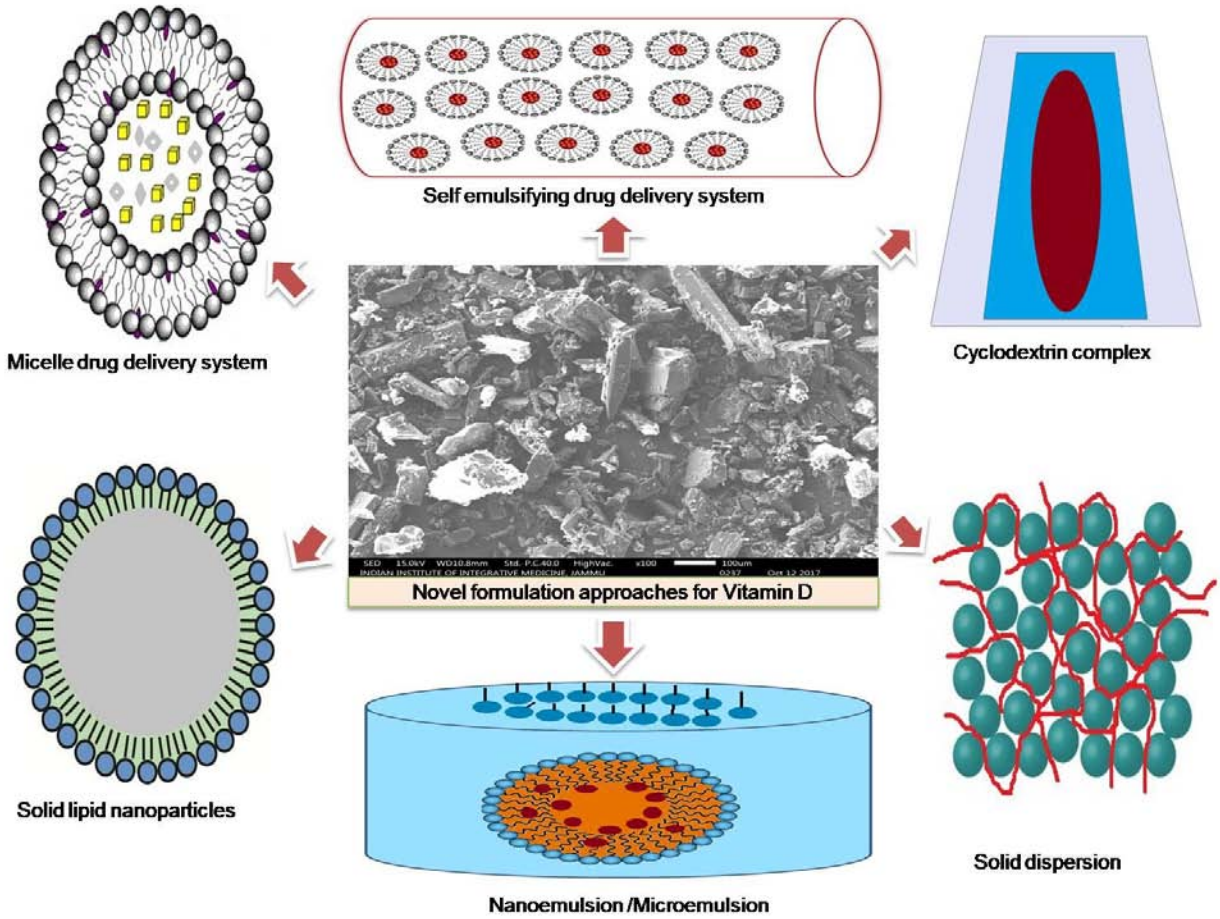

Fig. 1: Novel formulation approaches for cholecalciferol

The major source of vitamin D is exposure to sunlight. The deficiency of vitamin D could be attributed to limited UV exposure, topical application of sunscreen, certain sociocultural practices and urban life style. Secondary sources for vitamin D include dietary intake of foods naturally rich in vitamin D such as salmon, cod liver oil, sun dried mushrooms or vitamin D fortified foods [20]. In our country, availability, acceptability and cost of these dietary products limits their widespread use by the general population. This complex interplay between lack of adequate sun exposure, deficient intake and effective food fortification strategies makes Asian Indian population particularly susceptible to vitamin D insufficiency/deficiency. Thus, vitamin D supplementation in the form of pharmaceutical preparations is one of the most effective ways to prevent and treat vitamin D deficiency in high-risk groups [21].

3.1 Encapsulation of vitamins

Vitamins are sensitive molecules; therefore they should be preserved from harmful agents like heat and oxidants. Encapsulation is a promising and novel method for preserving the innate characteristics of vitamins over time. This process includes coating or trapping a biomaterial or a combination into another element. The entrapped substance is normally a liquid, while solid state substance can also be carried. The coating substance is known as capsule, wall material, membrane, or carrier. Micro/nanoencapsulation is defined as the creation of a barrier to inhibit unfavorable chemical interactions and for the controlled release of bioactive ingredients especially vitamins. Encapsulation offers a number of advantages including high intracellular uptake, potential to pass along the smallest body fenestrations, precision targeting, reduction in reactions between vitamins and surrounding medium, enhanced shelf life coated vitamins etc.

3.2 Novel delivery approaches for Cholecalciferol

3.2.1. Self-emulsified delivery system

SEDDS comprises of an isotropic mixture of drug, oil, surfactant and co-surfactant which upon oral administration gets emulsified in the aqueous medium in the GI tract. The oil phase of the SEDDS is generally selected from the group comprising, propylene glycol esters, medium chain mono-, di-, or triglycerides, long chain fatty acids, edible oils, or a mixture thereof. The non-ionic surfactants are selected from a group comprising polyoxyethylene products of hydrogenated vegetable oils, polyethoxylated castor oils or polyoxyethylene-sorbitan-fatty acid esters, polyoxyethylene castor oil derivatives or a combination thereof. The co-surfactants are selected from a group comprising polyethylene glycol chain lengths (preferably having a molecular weight of 200 to 600) and propylene glycol derivatives [22]. The self-emulsifying drug delivery system allows initial dissolution of the drug in oil phase with the concomitant addition of surfactant and co-surfactant, the whole formulation in final form remains as single phase liquid. The addition of excipients is so optimised that when introduced into an aqueous phase under gentle agitation, formulation emulsifies spontaneously to produce fine oil-in-water emulsions. The resulting oil-in-water emulsion is thermodynamically stable due to the relatively small volume of dispersed oil phase, narrow range of droplet size distribution and polarity of oil droplets. The oil-in-water emulsion shows higher absorption in the GI tract. The success of the self-emulsification in increasing the bioavailability of the drug depends on the oil-surfactant pair, surfactant concentration and the temperature at which self-emulsification occurs [22].

3.2.2. Solid dispersion

A solid dispersion (SD) is a dispersion system present in a solid form formed by dispersing a drug into a solid carrier. The drug is present in the carrier in a form of molecular state, colloidal state, a metastable state, microcrystalline state, and amorphous state, etc. The solubility and

dissolution rate of the drug present in an amorphous state (high energy state) are larger than those of the drug present in other crystalline states. The carrier is selected from the group consisting of hydrophilic polymeric carriers such as povidone, copovidone, hydroxypropyl cellulose, hydroxypropyl methylcellulose, cyclodextrins etc [23]. There are numerous formulations available in the market based on this technology due to its cheaper and reproducible qualities in terms of industrial perspective. The selection of carrier or mixture of the carrier for this approach is important. Neusilin and Soluplus are novel amorphous polymers developed for enhancing the solubility of the hydrophobic drug [23].

Table 1: Formulations of SEDDS/solid dispersion available in market

Delivery Platform	**Active moiety**	**Trade Name**	**Dosage forms**
SEDDS	Tretinoin	Vesanoid (Roche)	Soft gelatin capsule, 10 mg
	Isotretionin	Accutane (Roche)	Soft gelatin capsule, 10, 20 and 40 mg
	Cyclosporine	Panimum bioral (Panacea Biotec)	Capsule, 50 and 100 mg
	Cyclosporin A	Sandimmune (Novartis)	Soft gelatin capsule, 25, 50 and 100 mg
	Lopinavir and Ritonavir	Kaletra (Abbott)	Soft gelatin capsule,
Solid dispersion	Everolimus	Certican	Tablet
	Nabilone	Cesamet	Tablet
	Griseofulvin	Gris-PEG	Tablet
	Verapamil	Isoptin SR-E	Tablet
	Nivaldipine	Nivadil	Tablet
	Troglitazone	Rezulin	Tablet

	Lopinavir, Ritonavir	Kaletra	Tablet

3.2.3Solid Lipid Nanoparticles: Solid lipid nanoparticles (SLN) are spherical in shape with an average diameter between 10 to 1000 nanometers. Solid lipid nano particles possess a solid lipid core matrix that can solubilize lipophilic molecules. The lipid core is stabilised by surfactants (emulsifiers). The lipid typically includes triglycerides (e.g. tristearin), diglycerides (e.g. glycerol behenate), monoglycerides (e.g. glycerol monostearate), fatty acids (e.g. stearic acid), steroids (e.g. cholesterol) and waxes (e.g. cetyl palmitate). Various classes of emulsifiers (with respect to charge and molecular weight) have been used to stabilise the lipid dispersion [23]. The lipids have the intrinsic property of forming a core of hydrophobic drug by allowing adsorption over hydrocarbon chain and forming micelles with surrounding aqueous environment using polar groups of added surfactant. The whole structure is just like chylomicron which enhances the dissolution and allows passive absorption through a biological membrane. Regarding solid dispersed particles produced by using hydrophilic polymers, solid lipid nanoparticles are much more efficient in terms of biodegradability, non-toxicity, ability to incorporate hydrophobic drugs, chemical and physical stability, and controlled release of incorporated substances [23]. The dissolution of lipid material in aqueous media depends upon the concentration of surfactant added but there are certain lipids which are itself amphiphilic in nature especially lipid matrix containing high monoglyceride content and maximum drug loading can be achieved using such lipids.

3.3 Literature Survey

- **Luo Y et al, 2012,** prepared zein nanoparticles coated with carboxymethyl chitosan (CMCS) to encapsulate vitamin D3 (VD3). VD3 was first encapsulated into zein nanoparticles using a low-energy phase separation method and coated with CMCS simultaneously. Then, calcium was added to cross-link CMCS to achieve thicker and

denser coatings. The nanoparticles with CMCS coatings had a spherical structure with particle size from 86 to 200 nm. The encapsulation efficiency was greatly improved to 87.9% after CMCS coating, compared with 52.2% for that using zein as a single encapsulant. The physicochemical properties were characterized by differential scanning calorimetry and Fourier transform infrared spectroscopy. Nanoparticles with coatings provided better controlled release of VD3 in both PBS medium and simulated gastrointestinal tract. Photostability against UV light was significantly improved after encapsulation.

- **Teng et al, 2013,** developed complex nanoparticles from carboxymethyl chitosan (CMCS) and soy protein isolate (SPI) by a simple ionic gelation method. The effect of Ca2+ concentration, pH and CMCS/SPI mass ratio on the formation of nanoparticles was systematically investigated. Vitamin D3 (VD), a hydrophobic micronutrient, was successfully incorporated into the polymeric complex, forming particles with sizes between 162 and 243 nm and zeta potentials ranging from -10 to -20 mV. In comparison with CMCS, the CMCS/SPI complex required a lower concentration of Ca2+, and it showed better particle forming capability over a broad range of pH. These features resulted in an increased loading efficiency (6.06%). In addition, the complex nanoparticles achieved significantly higher encapsulation efficiency (up to 96.8%), possibly due to their compact structure and high capability of hydrogen bonding evidenced by Fourier transform infrared spectroscopy (FTIR). In contrast to the ones prepared with SPI, the complex nanoparticles exhibited a reduced (42.3% compared to 86.1%) release of VD in simulated gastric fluid and an increased (36.0% compared to 8.2%) release under simulated intestinal condition. These characteristics made the

CMCS/SPI complex nanoparticles an attractive candidate for the encapsulation and controlled release of hydrophobic nutraceuticals and bioactives.

- **Guttoff et al., 2015,** studied the influence of system composition and preparation conditions on the particle size and stability of vitamin D nanoemulsions prepared by spontaneous emulsification (SE). The influence of oil phase composition (vitamin D and MCT), surfactant-to-oil ratio (SOR), surfactant type (Tween 20, 40, 60, 80 and 85), and stirring conditions on the initial particle size of vitamin D nanoemulsions was studied. Nanoemulsions with small droplet diameters (d < 200 nm) could be formed using Tween 80 at SORε1 at high stirring speeds (800 rpm). These systems were relatively stable to droplet growth at ambient temperatures (<10% in diameter after 1 month storage), but unstable to heating (T >80°C). The thermal stability of the nanoemulsions could be improved by adding a cosurfactant (sodium dodecyl sulphate, SDS).
- **Park et al., 2017,** fabricated Nanostructured lipid carriers (NLCs) for encapsulating vitamin D3 (VD3), a lipophilic vitamin, by hot high pressure homogenization. The physicochemical properties of the VD3-NLCs were characterized, and the release profiles of VD3 in simulated gastrointestinal fluids were investigated. Optimum VD3-NLCs were obtained with a small diameter (132.9 nm), a high zeta potential (-41.90 mV), and a high encapsulation efficiency (85.6%). The stability of the VD3-NLCs was tested during 20 days of storage at 25 C under a wide range of pHs. In vitro digestion in simulated gastrointestinal fluids demonstrated their capability for controlled release because the NLCs were able to remain stable and protect the VD3 in simulated stomach fluid, but released more than 90% of the VD3 in simulated intestinal fluid.

- **Tipchuwong et al., 2017,** develops vitamin D3 fortified ice cream by incorporating vitamin D3 in an emulsified form using milk protein as emulsifier. Physicochemical stability of vitamin D3 emulsions using different milk protein emulsifiers including nonfat dry milk, sodium caseinate (Na-Cas), and whey protein isolate was investigated. Emulsion using Na-Cas had the smallest oil droplet size and the lowest creaming index throughout the storage time ($P < 0.05$) and was selected to fortify in full-fat, reduced-fat, and low-fat ice creams at 250 IU per serving. Vitamin D3 retention in each ice cream was determined after 0, 7, 14, 28 and 56 d of storage at -20°C.

4. Research Issues/gaps in the proposed area of work

Vitamin D sources are fairly limited in foods, therefore, there is a high demand to enrich food and beverages with vitamin D3. Cholecalciferol is highly sensitive to environmental stresses and can easily be oxidized, leading to loss of its functionality and physiological benefits. It is also reported to unstable at acidic conditions. Therefore novel formulation strategies are required for the efficient delivery of cholecalciferol. Cholecalciferol is available in the market, manufactured by conventional technologies of tablet formulation and capsules and such products are mostly suffered from low dissolution. Some investigations have been made to address these issues, still there is need to develop advanced formulations for efficient delivery of cholecaciferol.

5. Problem formulation

The deficiency of vitamin D is a global concern and a significant Indian population is deficient in this vitamin. The major source of vitamin D is exposure to sunlight but due to certain sociocultural practices and urban life style, the deficiency of this of vitamin D is compromised. Secondary sources include dietary intake of foods naturally rich in vitamin D. However, availability, acceptability and cost of these dietary products limit their widespread use by the

general population. Therefore, vitamin D supplementation in the form of pharmaceutical preparations is one of the most effective ways to prevent and treat vitamin D deficiency in the society. Vitamin D is susceptible to environmental factors and cholecalciferol is available in the market is manufactured by conventional technologies leading to limited bioavailability. Therefore novel formulations are required for efficient delivery of cholecalciferol.

6. Specific Research Objectives (to be accomplished during the course of this programme)

- Development of solid dispersion of cholecalciferol using polymer stable at acidic environment of the stomach (eg. HPMC Pthalate, Eudragit etc.).
- Evaluation of developed solid dispersion based formulation.
- Development of self-emulsified formulation of cholecalciferol.
- Characterization of developed self-emulsified formulation.

7. Contribution and need of the proposed Research objectives towards advancement in existing Research database

The deficiency of cholecalciferol is common in India. In north India, 96% of neonates, 91% of healthy school girls and 84% of pregnant women were found to be suffering from Vitamin D deficiency while in South India 40% of males and 70% of females were affected. The development of improved formulation with good bioavailability for vitamin D could be potential step in order to limit vitamin D deficiency. Vitamin D3 (Cholecalciferol) is a fat-soluble vitamin which appears as odorless white crystals with a very poor solubility (0.0013µg/ml). The stability of vitamin D3 is also one of the biggest issues since it readily gets oxidised and inactivated by moist air within a few days at room temperature. SEDDS as formulation approach can be used for dissolution enhancement. Further, solid dispersion could also possess potential for improved cholecalciferol delivery. These formulation strategies for

cholecalciferol offer several advantages such as high metabolic stability, enhanced solubility, high membrane permeability, improved bioavailability and long duration of action. The proposed research work is translational in nature and industry oriented and it could lead to an improved product for cholecalciferol.

8. Methodology to be adopted

1. Methodology and techniques

- **Chemicals required:** Required chemicals with suitable purity will be purchased from the reputed venders/company.
- **Solubility studies:** The solubility in various oils, surfactants and co-surfactant will be determined. An excess amount of drug will be added to each selected vehicle and stirred continuously for 72 h at fixed temperature. Finally, the mixture will be centrifuged for optimum period and supernatant will be filtered through a membrane filter and concentration will be determined by HPLC.
- **Self-emulsify drug delivery system of Cholecalciferol:** The selection of oil phase, surfactant and co-surfactant depends upon the solubility of the drug, pseudo ternary phase diagram and HLB value. Cholecalciferol will be dissolving into the oil phase and then subsequent addition of a surfactant and co-surfactant will be performed. The final dispersion will then encapsulated into capsules.
- **Solid dispersion formulation:** Cholecalciferol solid dispersion will be developed by using polymers which are stable in acidic environment of the stomach. Carriers in combination or individually will be used to form solid dispersion at different ratio of drug: carrier. The formed solid dispersion with enhanced solubilization will finally formulate into granules.

- **Evaluation parameters:**
  - **Morphological characterization:** Morphology of liquid samples will be determined by transmission electron microscopy and solid samples by scanning electron microscopy.
  - **Particle size and zeta potential:** Particle size and zeta potential are important factors affecting efficacy of the formulation, therefore these will be determined following suitable dilution of formulations into an aqueous media (using Zeta sizer). The value of zeta potential indicates the degree of electrostatic repulsion between particles in a dispersion. The high zeta potential provides stability of dispersion and prevents aggregation. Particle size of SEDDS will also investigated in acidic pH.
  - **In vitro dissolution studies:** Dissolution will be determined using USP Type 2 paddle type apparatus in 900ml 0.1N HCl and pH 6.7 phosphate buffer maintained at 37±0.5^{o}C and 75rpm. 50µl of the sample will be withdrawn at predetermined intervals which will be compensated by the further addition of fresh dissolution medium into the flask. The sample will then estimate for cholecalciferol using HPLC. The effect of digestive enzyme on dissolution will also be evaluated.
  - **Differential Scanning Calorimetry (DSC):** DSC thermograms of pure drug and formulation will be determined by heating samples at 10^{o}C per minute.

- **X-ray diffraction:** XRD pattern of pure drug and formulations will be determined by running diffractograms at a scanning speed of 80 cycles/min over a 2Θ range of 0^o to 80^o.
- **HPLC estimation of cholecalciferol:** Cholecalciferol solution 20µg/ml will be prepared by dissolving in n-hexane and methanol (1:9 v/v). The analysis will be carried out at room temperature using 15 cm column, mobile phase as methanol-acetonitrile (7:3 v/v) and 50µl as the injecting volume at room temperature of 25^oC with a flow rate of 1.5 ml/min. The final measurements will be made at a wavelength of 265 nm.
- **Pharmacokinetic evaluation:** Wistar male albino rats (120 – 125 g) will be maintained for 10 days on vitamin D deficient feeding with free access to water. They will be housed individually in stainless steel metabolism cages, which permit collection of urine and faeces. All animals will be fed with a formulation of cholecalciferol administered via oral gavage. Serial blood samples from rats will be collected pre-dose, and at 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 4.0, 6.0, 8.0, 12.0, 24.0, 36.0 and 48.0 h post-dose directly into heparinized vacutainer tubes and immediately centrifuged to separate plasma and red blood cells (RBC). In a separate study, urine and faecal samples from remaining three rats will also be collected at an interval of 0-6, 6-12, 12-24, 24-36 and 36-48 h post dose. Plasma and urine samples will be analysed for 25(OH)D concentration after protein precipitation with methanol using HPLC. The maximum concentration of the drug in serum (Cmax) and the time to reach it (Tmax) will also be determined.

Subsequently, AUC, half-life, the volume of distribution, clearance and elimination rate constant will also be determined. The effect of some parameter eg. pH, fatty food on bioavailability/bio-accessibility will be evaluated and biochemical parameter will be noted.

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10. Place of Work: Maharaja Ranjit Singh Punjab Technical University Bathinda

11. Resources (Hardware/Software/) required & availability status: All required glasswares,

chemicals and equipment are available at the place of work.

12. Extent of External Assistance Required (if any):N.A.